

A STUDY OF THE GRANULAR
AND ATYPICAL FORMS OF
SPIROCHAETA PALLIDA
IN TISSUES

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Science,
in the University of Michigan.

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Investigation conducted under a grant from the
Committee on Syphilis Research



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MANY methods for the demonstration of the spirochete of syphilis in tissues have been suggested since Bertarelli, Volpino, and Bovero¹ described a silver nitrate impregnation in 1906. These stains all depend on the use of the silver salt in varying dilutions, the only changes being in the preliminary treatment of the tissue and in the reduction or precipitation following. Some of these methods were applied to selected tissues and the results compared not only from the standpoint of diagnosis but also from that of the study of the disease process.

The material used was from three congenital and three acquired cases of syphilis. The first tissue was from a congenitally syphilitic liver and had been used in the department as control material for twenty years. It was fixed in formalin and preserved in ethyl alcohol. The second case was lung and showed white pneumonia. It had been kept two years in alcohol after fixation in formalin. The third like the first was a liver from a congenitally syphilitic fetus. These tissues all contained spirochetes in large numbers, but the third one gave a different staining reaction as it had been preserved in formalin rather than in alcohol.

One of the tissues from the adult syphilitic cases was removed at operation while the other two were autopsy material. The operative tissue consisted of a pair of tonsils from a woman aged twenty-five. These had been removed, not with the suspicion of syphilis, but for simple enlargement. No history of antisiphilitic treatment was recorded. The tissue was fixed in formalin and blocked in paraffin from which single sections were cut. One of the post-mortem cases was that of a male, age fifty-two, who was found dead. He showed active syphilis of the meninges, aorta, and heart. Tissue from the aorta, fixed in formalin and embedded in paraffin, was used. No series of syphilitic cases would be complete without

*Work carried out under a grant from the American Committee on Research in Syphilis and published with its approval. From the Pathological Laboratory of the University of Michigan, Ann Arbor.

¹Bertarelli, Volpino, and Bovero; *Centralbl. Bact., Bd. XL u. XLI, 1906.*

one of tabes or paresis. A white male, age thirty-two, left his home one day, and the next was found in water with only his head showing. In addition to death by drowning the postmortem diagnosis included syphilis of the brain and aorta. Twenty-four hours must have elapsed between death and the fixation of the tissue in formalin in which liquid it was also preserved.

The methods used for the purpose of this comparison may be classified under two headings: first, the impregnation of entire blocks of tissue from which single sections are later cut, and second, the staining of single sections cut on the freezing microtome or from paraffin blocks. The three methods of block impregnation employed were those of Levaditi, Haythorn, and Jahnel. Six stains for single

	1	2	3	4	5	6
1-2-3 CONGENITAL						
4-5-6 ACQUIRED						
G=GOOD P=POSITIVE						
N=NEGATIVE						
LEVADITI	P	P	P			N
HAYTHORN	G	N	P			N
JAHNEL	P	G	G			G
WARTHIN-STARRY	P	P	G	P	P	N
IMPROVED WARTHIN-STARRY	G	G	G	P	G	N
DIETERLE	P	G	G	G	P	G
WRIGHT	P	P	P	P	N	N
GYENES-STERNBERG	G	P	P	N	P	N
ARMUZZI-STREMPER	P	P	G	P	P	P

Fig. 1.—A tabulation of the results obtained by the various staining methods.

sections were tried. These were the Warthin-Starry, the improved Warthin-Starry, Dieterle, Wright, Gyenes and Sternberg, and Armuzzi and Strempel. Of the above methods the Warthin-Starry with its modifications and Dieterle are most frequently used in this laboratory; therefore, all things being equal, these would be expected to give the best results. The Levaditi and Wright stains are often used but the remaining four were done especially for the purpose of this comparison and were in a measure handicapped.

The procedures outlined for the block impregnations were carefully carried out with the single exception that the tissues which had been preserved in alcohol were placed in formalin for twenty-four hours before starting. Cover-glass preparations, cut from paraffin blocks and mounted without the use of albumin fixative, were



Fig. 2.—*Spirocheta pallida* in white pneumonia stained by Levaditi's method. Magnified 2,700 times.

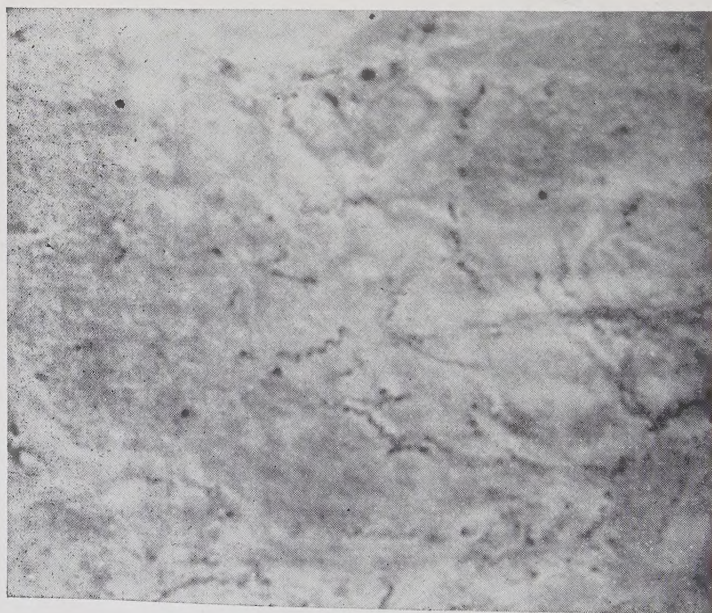


Fig. 3.—Haythorn's stain of congenital syphilis of the liver. Magnified 2,700 times.



Fig. 4.—Single spirochete of syphilis in the brain. Jahnel's uranium method. Times 2,700.

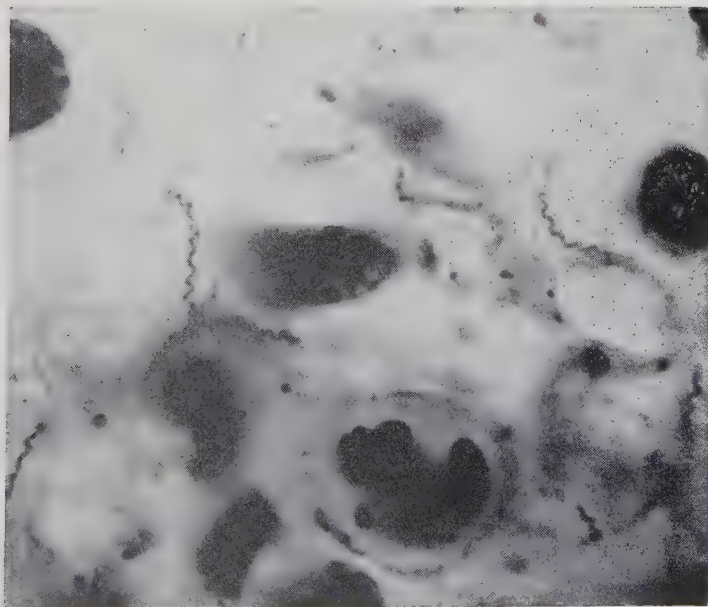


Fig. 5.—Congenital syphilis of the lung by the Warthin-Starry method. Magnification of 2,700.

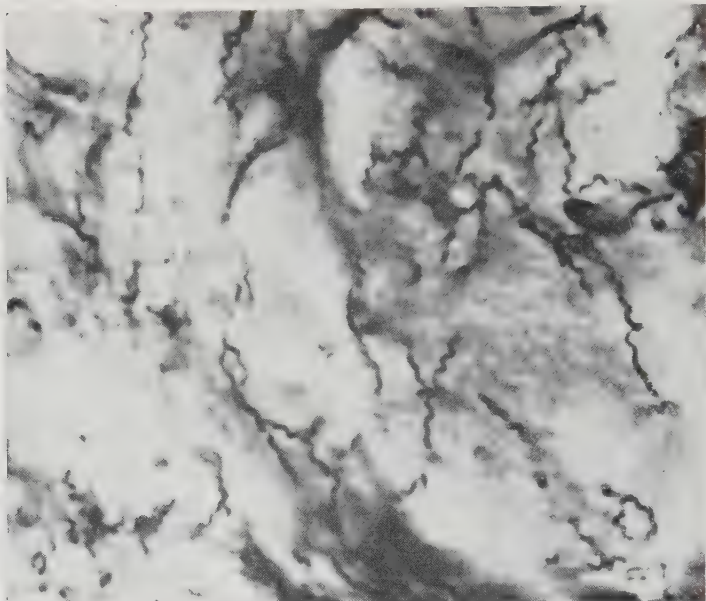


Fig. 6.—Fetal liver preserved in alcohol for twenty years. Improved Warthin-Starry stain. Times 2,700.

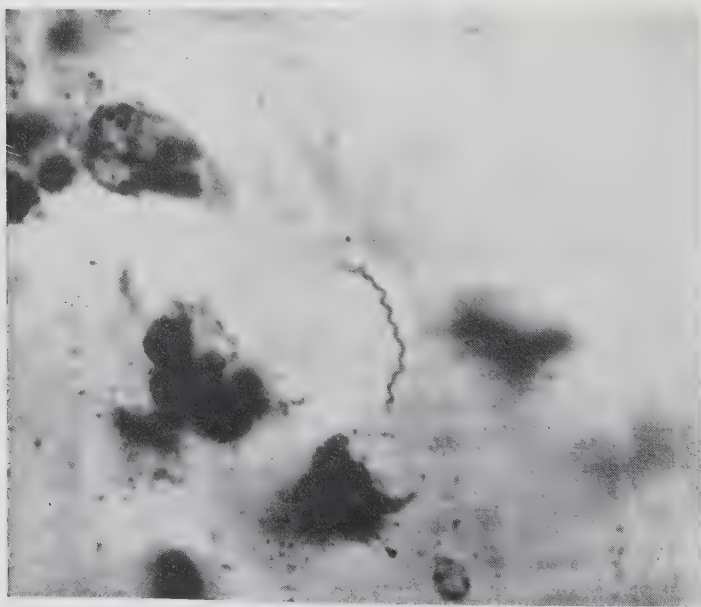


Fig. 7.—Acquired syphilis of the aorta. Single spirochete by the improved Warthin-Starry impregnation. Times 2,700.

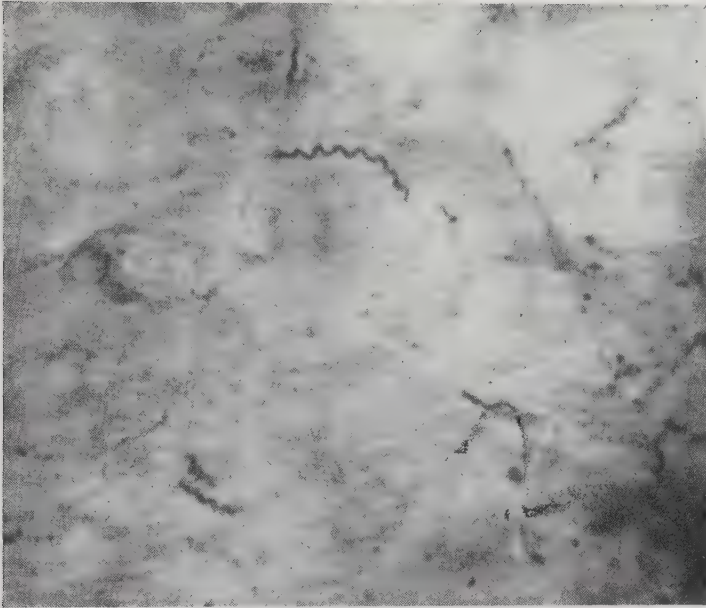


Fig. 8.—Spirochetes in the tonsil by the gum method of Dieterle. Magnified 2,700 times.

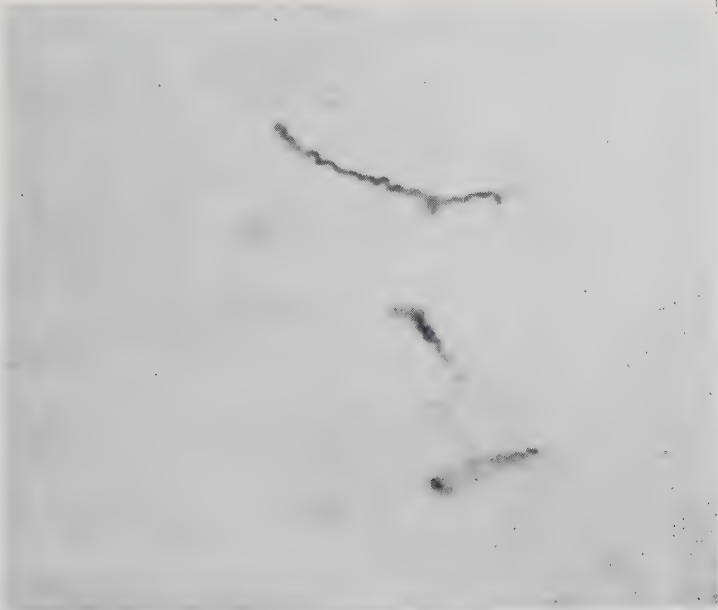


Fig. 9.—Enormous spirochetes of syphilis in the brain of a paretic. Dieterle's stain; times 2,700.

used for all the single section methods, though several were primarily designed for frozen material.

To designate the degree of satisfaction given by the various methods in their application to each of the tissues the following nomenclature was employed: negative, if no spirochetes could be seen; positive, if the organisms were visible but stained poorly or with little contrast between them and the tissue; good, if the majority of the organisms were well stained and stood out from the background so that photographs could be readily made. The histologic differentiation of the tissue elements is also considered under the individual stains.

Due to lack of stock material only the three congenital cases and that of paresis could be used for the block staining methods. Levaditi's method gave positive results in the three congenital cases but the brain blocks were negative. When the material preserved in alcohol was not first treated with formalin the tissue was irregularly stained and only a small fraction of the organisms could be seen though a few of these were thickly coated with precipitated silver. With the preliminary formalin treatment the tissues were a uniform pale yellow and almost all the spirochetes were stained, but only lightly.

Haythorn's impregnation gave good results in the twenty-year-old congenitally syphilitic liver, positive results in the lung tissue, but negative in the recent liver material and in the brain. This method and Levaditi's present a background in which the tissue elements are not suitably stained for the study of the body reaction in relation to the spirochetes.

Of the block impregnations Jahnke's uranium nitrate method gave by far the best results. Of the three congenital tissues all but the first liver, which was positive, gave good results. The spirochetes in the brain were very well demonstrated as this method is especially suited to material from the nervous system. The tissues were stained better than in either Levaditi's or Haythorn's methods but still left much to be desired. Were it not for the extreme length of time necessary to complete this method it would be most satisfactory in the given tissues.

The Warthin-Starry method, which has been used in this laboratory for eight years, gave good results in the white pneumonia material and was positive in all the other cases except the brain in which it was negative. It is difficult by this method to get a strong

contrast between organism and host especially if the material has been in alcohol for any length of time. This as well as the improved Warthin-Starry method gives a good stain of the tissue elements.

The improved Warthin-Starry method gave good results in the congenital cases and in the aorta, but was only positive in the tonsil section, and in the brain was negative. The short length of time required and the convenience with which the cover-glass method works with the routine diagnosis from paraffin sections gives a very great advantage over the older block methods and the more recent frozen section technic. This method is quite dependable in any material other than that of the central nervous system.

Dieterle's stain gave good results in the two more recent congenital cases and in the tonsil and brain, but was only positive in the oldest case of congenital syphilis and in the aorta. It was fortunate that in the aorta the spirochetes were not confined to the necrotic areas, as is often the case, but also were in the areas of cellular infiltration where it is possible for this method to demonstrate them. For brain sections this is the best rapid method, being more certain and less difficult in the technical procedures than that of Armuzzi and Strempel which it closely resembles. The tissue is almost colorless by this method making it desirable for photography but poor for study.

Wright's method is very rapid but does not stain all the spirochetes and rarely gives the heavy impregnations so essential to photography. The tissue is stained yellow with almost no histologic differentiation. All the congenital cases gave positive results but the tonsil was the only one of the adult tissues to do so, as the aorta and brain were negative.

Gyenes and Sternberg's method was the oldest one for single tissue sections that was attempted. It gave good results in the first liver case and was positive in the other two congenital tissues and in the aorta, but was negative in the tonsil and brain. It is a rapid method but is not as good as the more recent variations.

The method of Armuzzi and Strempel is much better suited to frozen sections than to cover-glass preparations as it is necessary to stir the sections at one point which is difficult if they are attached to number one cover slips. This stain gave good results in the white pneumonia material and was positive in all the others. The background is not suited to study of the tissue.

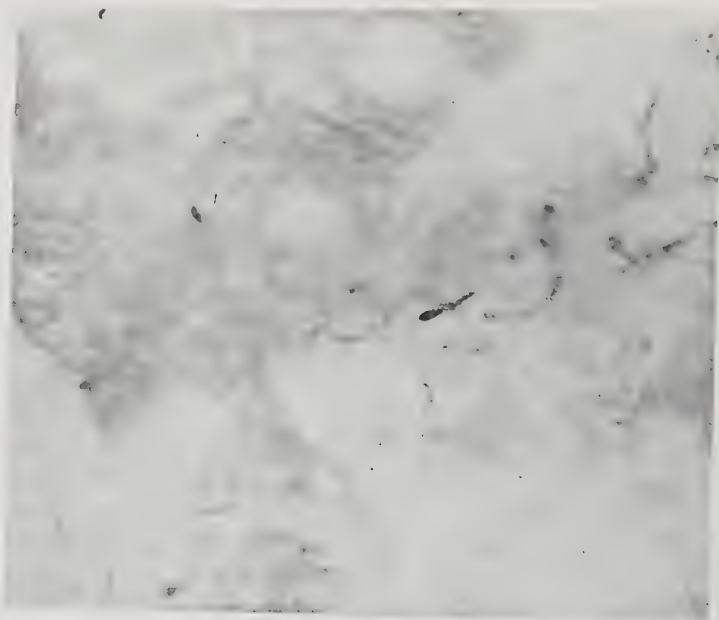


Fig. 10.—Congenital syphilis of the liver by Wright's method. Times 2,700.

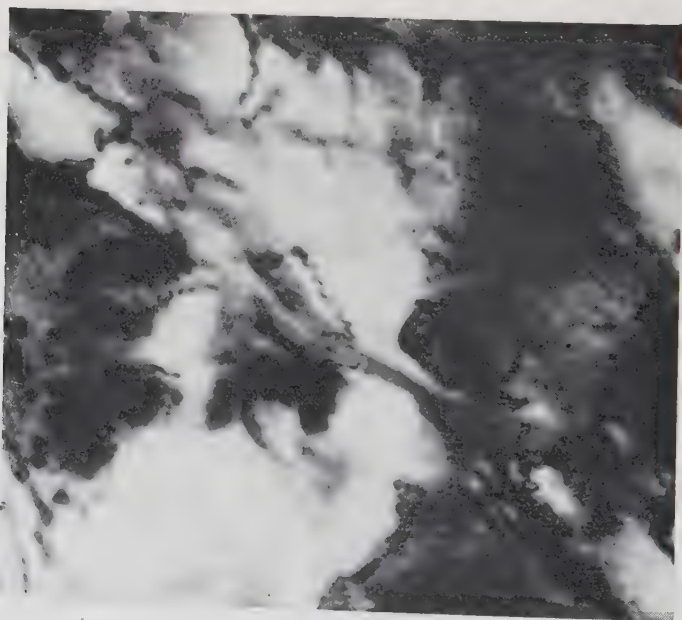


Fig. 11.—Congenital syphilis of the liver by the impregnation of Gyenes and Sternberg. Times 2,700.

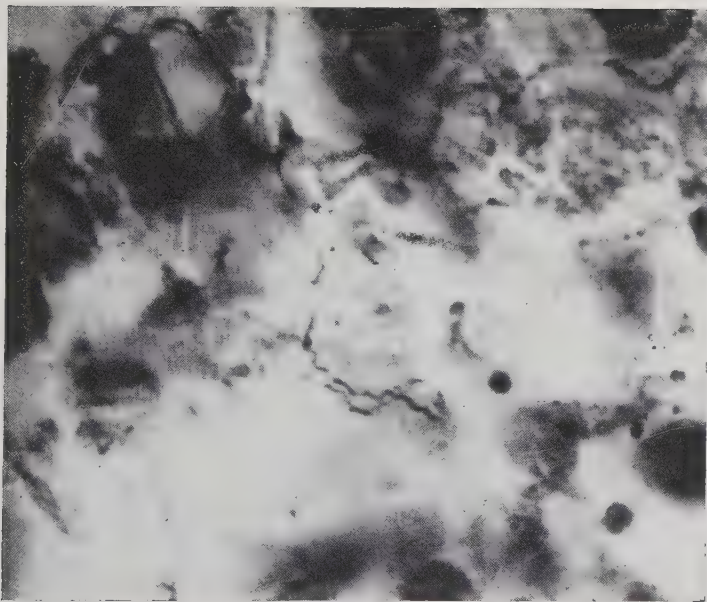


Fig. 12.—Syphilitic fetal lung stained by the method of Armuzzi and Strempel.
Times 2,700.

From the above records it is evident that the congenital material stains readily by any method, but gives the most satisfactory results with those of Jahnelt, Warthin-Starry, and Dieterle. Dieterle's method was also best for the tonsil tissue and the brain, though the latter gave good results with the long method of Jahnelt and was positive by Armuzzi and Stempel. In the aorta the improved Warthin-Starry method was the only one to give good results, and was certainly the only one by which any considerable percentage of the organisms was demonstrated. In consideration of the frequency with which the aorta is affected in syphilis, the improved Warthin-Starry method is of no small value. In addition to getting more rapid results, if there is little tissue, it is much better to embed it and cut sections which can be stained by various methods than it is to place all the confidence in a single block impregnation. No stain for the demonstration of *Spirocheta pallida* in blocks or single sections has reached perfection, but by selecting a method or methods suited to the given tissue it is almost always possible to find the organism if it is present.

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(Received for publication, September 22, 1930.)

IN A PAPER on "The Excretion of Spirocheta Pallida Through the Kidneys" (J. Infect. Dis. 30: 569, 1922), one of us described the characteristic degenerative changes occurring in Spirocheta pallida in the renal tubules. In granular casts spirochetes were present, either apparently unchanged or showing various stages of disintegration. Desquamated epithelial cells containing single or numerous organisms were common in the lumina of the tubules. In the phagocytes the spirochetes were often found coiled into a loop, or circle, or horse-shoe form, which apparently gradually contracts until it forms a round granule still taking the silver impregnation. All transition stages of this phagocytosis and destruction of the organism were shown. The great majority of the organisms reaching the collecting tubules showed signs of disintegration. Granular and beaded spirochetes were numerous in the lumina. The destruction of the organism appeared to take place chiefly in the lumen of the tubules, and the conclusion was drawn that but few preserved organisms could be found in the urine.

*Investigation conducted under a grant from the Committee on Syphilis Research.

Since this observation, we have been much interested, and not a little intrigued, by the almost constant association of granular silver-staining forms with perfectly preserved spirochetes in the lesions of latent syphilis, particularly in focal necroses of the aortic wall, usually in the media bordering on the intima, and always associated with characteristic lymphocyte and plasma-cell infiltrations around or along the vasa vasorum of the media and adventitia. Numerous polymorphonuclear cells are found in and about the borders of these focal necroses, and their nuclei usually show karyorrhexis or pyknosis. The necrosis is of the caseous type; the foci are sharply circumscribed and somewhat oval in shape, the long axis parallel with that of the aorta. These necrotic foci may be found in any part of the aorta, but are more frequently found in the ascending and transverse portions of the arch. They are usually few in number and may not be found in the milder forms of syphilitic aortitis. They develop in the media, often extending to the intima, and but rarely to the adventitia. In sections stained with haematoxylin and eosin the necrotic area appears bluish in contrast to the pink of the surrounding tissue, due chiefly to the diffused chromatin of broken-down nuclei, and in some cases to an early impregnation with lime salts. The elastic tissue and muscle disappear from the area; the polynuclear cells are replaced by mononuclears, and the necrotic material is gradually replaced by hyaline scar tissue. Usually these focal necroses are of small size, varying from one-half to several millimeters in length, and from one-tenth to half a millimeter in depth. They are usually not visible to the naked eye. They are often very irregular in shape, and are not pyramidal, as might be expected from the blocking of the vasa vasorum. They are often irregularly branching in shape with extensions between the elastic tissue bands for some distance. The lesions are always more sharply circumscribed on the adventitial side, and are more diffuse on the intimal border where they may be in approximation to atheromatous plaques. While some of them appear to be necrotic miliary gummas, others represent a focal necrosis of the aortic tissues themselves, infiltrated with polynuclear and mononuclear cells, but without any granulomatous formation. They do not appear to be infarcted areas due to the blocking of the vasa vasorum, although the latter may be a contributory factor, as the vasa vasorum of the portion of the aorta involved always show marked perivascu-

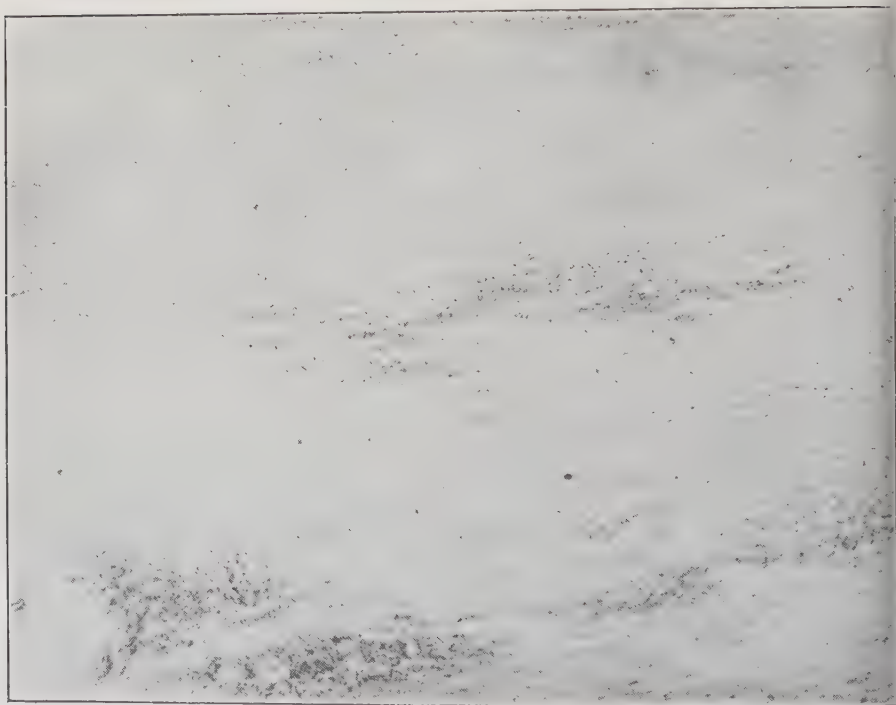


Fig. 1.—Low power view of focal syphilitic necrosis in media of aorta. Latent syphilis.



Fig. 2.—High power view of border of focal necrosis in media of aorta in latent syphilis. Karyorrhexis of nuclei and diffusion of chromatin.

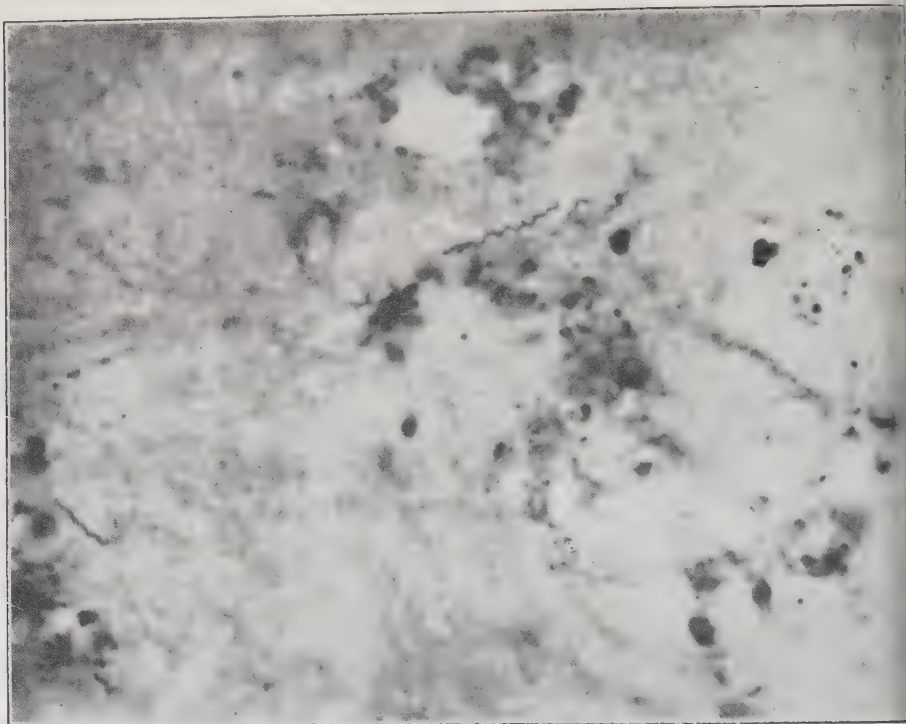


Fig. 3.—Typical *Spirocheta pallida* from border of focal necrosis in aortic media seen in previous figures. X 2500.

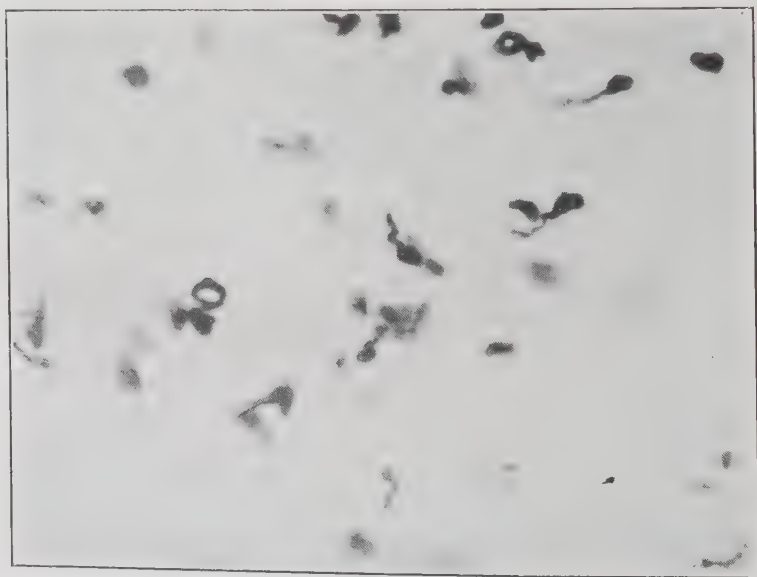


Fig. 4.—Atypical spirochete forms from central portion of focal necrosis. Numerous forms with terminal knob. Contracting loop forms. X 2500.

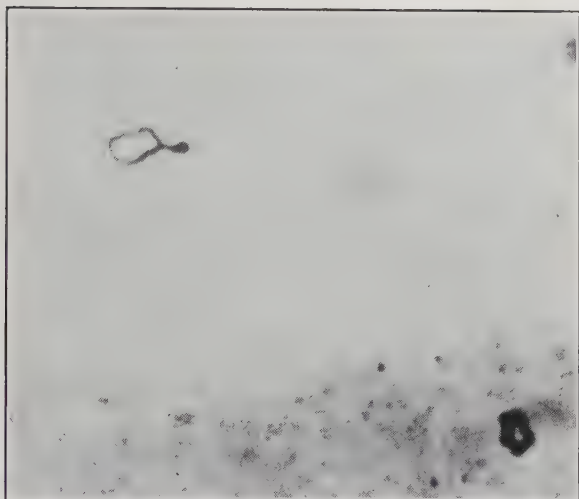


Fig. 5.—Two stages of loop formation and contraction of spirochetes from area of focal necrosis in aorta. X 2500.



Fig. 6.—Contracting loop-forms of spirochetes from same lesion as the preceding. X 2500.

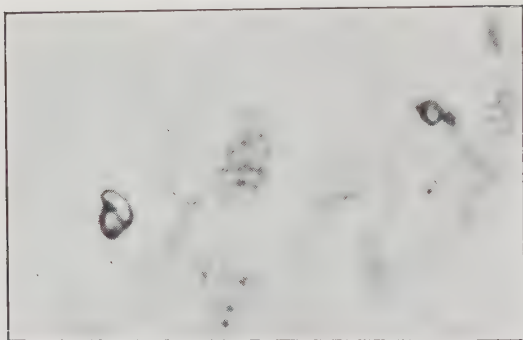


Fig. 7.—Two characteristic contracting loop-forms of spirochetes from same area of focal aortic necrosis. X 2500.

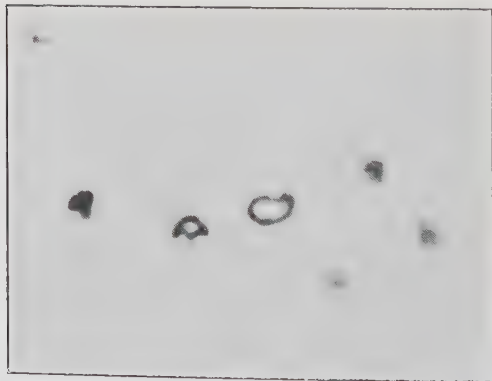


Fig. 8.—Loop and granular forms from same lesion. X 2500.

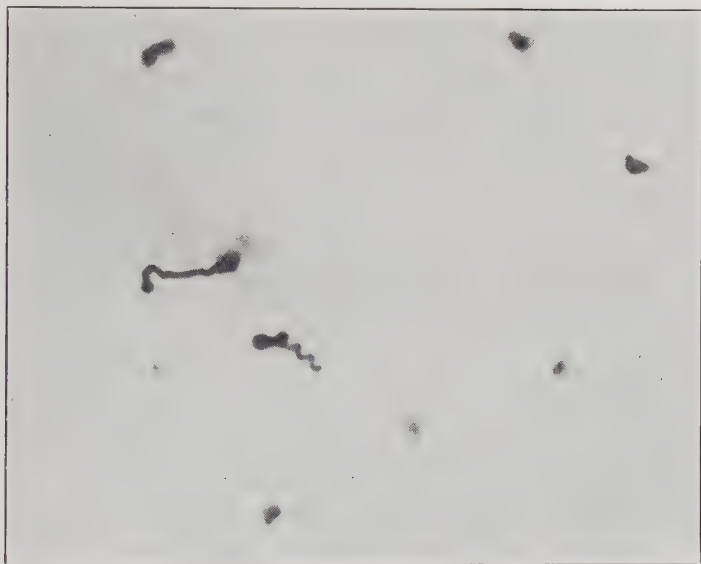


Fig. 9.—Two short spirochetes with knobbed ends, and granular forms from same area of aortic focal necrosis. X 2500.

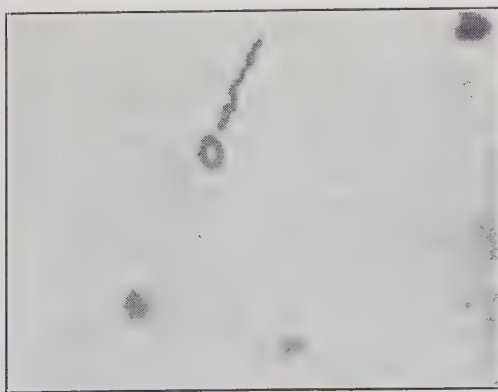


Fig. 10.—Typical short spirochete and contracted loop-form, from center of aortic focal necrosis. X 2500.

lar infiltrations and the arterioles more or less thickening and obliteration. What is of the greatest interest is that we can always demonstrate typical spirochetes about the borders of these focal necroses, even when we can find none in the perivascular infiltrations. The polynuclear reaction resembles that seen in some cases of congenital syphilis and in active syphilitic myocarditis. The spirochetes are usually most numerous between the necrotic area and the intima. They usually lie parallel with the tissue layers of the aortic wall, but around the capillaries are found usually lying at an angle to the elastic fibers. In the interior of the necrotic foci typical spirochetes are found but rarely. They are replaced by atypical forms of various sizes and shapes showing all possible transition stages from a typical spirochete to fine single granules almost submicroscopic in size. A definite cycle of transformation is apparent. The typical forms do not break up into multiple granules or beaded forms. The first stage is apparently the development of a knob, usually at one or both ends, but occasionally in the middle of the organism; the ends then bend together, forming a horse-shoe loop, this in turn becomes an irregular circle, which contracts into a solid irregular granule, finally becoming a single, small, rounded granule. The loop stage does not invariably appear, as some organisms, after the appearance of the knob-like extremities, change into elongated amorphous masses without any central opening, and contract as do the loops until the minute granule is all that remains. A submicroscopic form following the minute granule is inferred, but we are not ready to offer positive demonstration of it at the present moment. These degenerative forms, with the same apparent cycle of transformation to the granular stage, have been present in all of the necrotic foci found in luetic aortas. The number of atypical spirochetal forms varies very markedly, but is usually proportional to the size of the lesion. The degenerative forms are invariably present, even when typical spirochetes may be apparently absent.

The atypical forms take the silver impregnation as readily, or even more so, than do the typical spirochetes. They may be demonstrated with solutions of silver nitrate varying from one one-hundredth of 1 per cent and from P_H 3.5 to P_H 4.5 (water adjusted with 2 per cent citric acid solution and color indicator). The original Warthin-Starry method, or any of its modifications, serves to

show the granules, and we have also shown them by the methods of Levaditi, Armuzzi and Stempel, Dieterle, and Wright's silver stain. The recent modifications of the improved Warthin-Starry method give the most uniform results, and the best picture of the various stages. That the different stages of the forms observed represent changes in the spirochetes themselves cannot be doubted, owing to

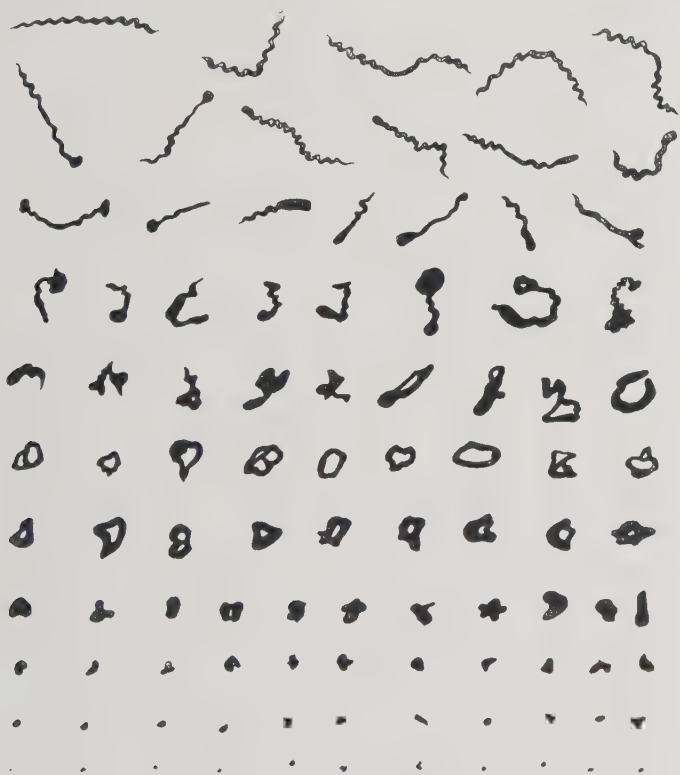


Fig. 11.—Drawing showing typical forms of spirochetes from border of aortic focal necrosis, and all stages of transformation to granular forms.

the specificity of the staining method, and the transitional stages from the perfect spirochete to the minute granule.

The significance of this transformation and of the final granular stage remains to be determined. From their presence in the necrotic aortic foci, as well as in the renal epithelium and tubules, they might be regarded as purely degenerative forms, but if so, the problem of the viability of the final minute single granules remains to be

proved or disproved. To settle this problem experimental proof will have to be obtained, and such demonstration will be difficult. It is easy to conjecture that the process is a reproductive cycle and that the minute granules represent the infective stage of the organism, but no positive proof exists for these hypotheses, and statements to that effect would be premature guesses. We can only say, at the present moment, that there appears to be a regular cyclical transformation of *Spirocheta pallida*, under certain conditions, to a minute granular form, the relation of which to the typical spirochete form remains to be determined. We are aware of the fact that various other workers have seen at least certain stages of this transformation and hold that the granular form represents a definite stage in the life-history of the spirochete, but we do not consider that any of the evidence so far offered for this conclusion is convincing, inasmuch as hypothetical considerations, rather than experimental, form the basis by which it has been reached. In the meantime this laboratory is concerning itself with this larger problem, approaching it from all angles, in the attempt to prove or disprove the hypothesis that the granular form represents a definite life-stage of the spirochete. Whether involution or evolution is the significance of these atypical spirochete forms remains to be determined.

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THE most important problem confronting students of syphilis to-day is that concerned with the question of a growth cycle of *Spirocheta pallida*, and the relation of different stages in this cycle to the histologic lesions, as well as to the serologic and clinical manifestations of the disease. In a previous paper¹ we have described looped, ring and granular forms of spirochetes found particularly in focal aortic lesions. At that time we concluded that there appears to be, under certain conditions, a regular cyclical transformation of *Spirocheta pallida* to a minute granular form, the relation of which to the typical spirochete form remains to be determined. The question as to the significance of these atypical spirochete forms, whether involution or evolution, was left open.

Similar granular forms were described by Manouelian in old gummas and other late lesions.² He regarded these forms as representing a transmutation series from the typical spirochete form to a minute corpuscle which can pass through a filter. These atypical granules are much more numerous than the typical spirochetes, and are very abundant where the latter are rare or cannot be demon-

strated at all. He regards the presence of these granules as confirmatory of the syphilitic nature of a late lesion, even in the absence of typical spirochetes. Levaditi³ confirmed the work and conclusions of Manouelian. He describes stages leading from the spirochete to the granules, the ultimate granules being from 0.1 to 0.3 microns in diameter. He believes that these findings might explain late syphilis without spirochetes, paresis without spirochetes, and finally malignant syphilis. The resistant forms are not sensitive to the chemicals that kill the vegetative (spirochete) types. He further believes that an ultramicroscopic form represents part of the evolutionary cycle of *Spirocheta pallida*. His illustration presenting the various stages between spirochetes and granules shows that he is dealing with the same forms described by us, as occurring in necrotic lesions in the aortic wall. In an earlier paper⁴ Levaditi and associates reported observations made on the inoculation of tissue-grafts containing spirochetes into mice. He found that in such grafts the spirochetes disappeared within forty-eight hours and that none could be found after thirty-six days. However, grafts from those inoculated lesions devoid of spirochetes, when further inoculated into rabbits, produced chancres laden with spirochetes. Levaditi, therefore, draws the conclusion that in the mouse the spirochete survives and remains virulent in an ultramicroscopic granular phase, which is a part of the evolutionary cycle.

As to the various atypical forms of *Sp. pallida* that have been described by various investigators, whether regarded as involution forms or as representing cyclical stages, but few of these investigators have been able to interpret their findings in terms of extra- and intracellular phases. Many of those who have studied spirochetes in stained sections agree that the organisms may be found within or without the cell, and that appearances occasionally noted suggest that the spirochetes are passing through the cell wall. Levaditi⁵ and associates have described atypical forms, small spirochetes and granules as appearing within certain cell types apparently derived by involution from the typical spirochete form. McDonagh⁶ evolved a rather complicated sexual cycle out of extra- and intracellular forms which he demonstrated by specific methods in infected tissues, particularly chancres and lymph nodes. Meirowsky⁷ worked out his theory of spirochete reproduction by means of detailed descriptions of his findings among free organisms, but had little to say as to the

location of reproductive stages in human tissues. Other authors have also presented theories of life-cycles of *Sp. pallida*, giving diverse descriptions of the stages involved, but as yet none of these theories has met with general acceptance, and the interpretations of the findings given by these authors have been called into question.

As a result of the intensive study of *Sp. pallida* in its relation to tissue lesions which we have carried out for a number of years, we have come in this laboratory to distinguish certain class or type-forms, which staining by our specific staining methods, we regard as representing different stages of *Sp. pallida*. These forms we tentatively term the: (1) typical large spirochete form; (2) the ring or polymorphous form; (3) the small spirochetes; (4) the lymphocytic granules; and (5) the giant cell reaction stage granules. In this paper we shall consider these forms as we have studied them in the chancre, bubo, and late aortic lesions.

"RING FORM." POLYMORPHOUS FORM

In our previous paper we concerned ourselves with the extracellular ring and granular forms of *Sp. pallida* found especially in focal necrotic areas in the aorta. These have also been described by Levaditi and Manouelian. We noted that all forms between the typical large spirochete and the small granular form exist, the intermediate forms usually being ring shaped. In addition to their presence in aortic necroses, we find similar ring-shaped forms in chancres, buboes, secondary skin lesions, and in late lesions in the heart, aorta, and skin. They are found, not only in necrotic areas, but in the perivascular plasma cell and lymphocyte infiltrations, both in association with typical large spirochetes and without them. They are both extra- and intracellular. The demonstration of ring forms in latent syphilitic perivascular lesions, in which no typical spirochetes can be found, was the first important link of the chain to be demonstrated by us. The number of ring bodies is not directly or inversely proportional to the number of typical spirochetes present. When the latter are also present, they may frequently be seen connected to the ring forms, the spirochete being extracellular but passing through the cell wall to the intracellular ring-form mass. There is an appreciable change in the staining reaction at the point where the spirochete is connected with the ring form, which in part accounts for the fact that this form has been overlooked by other observers. The large

spirochete form may also be connected with the ring body by a fine spiral thread which appears to be of the small spirochetal type.

The term "ring form" is not strictly accurate, as it does not apply to all of the intracellular spirochetal masses showing the same staining reaction. Many of these are irregular in form, others are stellate. The latter under proper staining technic often show numerous small spirochetes growing out from them, forming a veritable Medusa head within the cell. There are also oval and elongated types of the basic ring form, which may extend along the supporting framework of the tissue, and when stained contrast markedly with the uninvaded areas.

The cell type in which the polymorphous form is found varies in different tissues and in different parts of the same tissue; but most commonly the endothelial cells of the capillaries and the cells of the supporting framework of the tissues are found to contain them, while they are absent from other cell forms. In the inguinal bubo, in which the ring forms are especially numerous and prominent, each endothelial cell in the infiltrated areas may contain one, at least; and all stages of the spirochete cycle are present. In well-stained sections the polymorphous forms may be readily identified at a magnification of one hundred, as they are more bulky than the ordinary spirochetes and form a strong contrast against the pale yellow or colorless background. They may vary in size, as well as in shape, and there may be much smaller ring forms present along the connective tissue cells, but even these show their identity, not only by their specific staining reactions, but by their relation to the small spirochete forms. While the polymorphous forms are found chiefly in the endothelial system and supporting tissues, they also occur as very small ring forms occasionally in lymphocytes, and less frequently in other cells, apparently as intermediate stages between the granule form and the large spirochete, when the latter is present in the lesion.

The extracellular ring forms apparently arise through the destruction of the parasitized cells containing them, liberating the intracellular ring form, and giving it the same staining reaction as the large typical spirochete forms which then appear, and which, therefore, represent an extracellular phase of the intracellular form. The ring forms previously described by us, in our paper on focal necroses in aortic syphilis, were limited to the areas in which cell necrosis had taken place; but such polymorphous forms may be found in any part of the aortic wall in which active lesions of syphilis (perivascular

infiltrations) are present. In such lesions they are frequently associated with the small form of spirochetes. It must be repeated here that a special staining technic is necessary for their demonstration, as the usual staining methods applicable for the demonstration of the large spirochete forms will not reveal them.

SMALL SPIROCHETE FORMS

In our previous studies on spirochetes we had frequently noted the occurrence of atypical small spirochetal forms, more or less tenuous and filamentous, and possessing but few turns (2-4). One of us (Warthin) has always rejected these in the diagnosis of the syphilitic nature of a given lesion, and has rigidly adhered to a criterion established by the large spirochetal form with close, tight turns. Up to the present time all of Warthin's studies in syphilis have been based upon the demonstration of the typical large form of *Sp. pallida*. In only about 50 to 60 per cent of cases showing identical tissue lesions could these typical large forms be demonstrated, and in some cases their number was so small as to be wholly out of proportion to the magnitude of the lesions present in the tissues. These discrepancies were for a long time thought to be due to the inadequacies of our staining methods or to imperfect fixation of tissues, or to postmortem or extravital changes in the spirochetes contained in the lesions. Modifications of our staining technic (Olsen), however, show such frequent occurrence of small spirochetal forms in active syphilitic lesions that we are now constrained to accept them as representing stages in the life-cycle of *Sp. pallida*. We are, therefore, now disposed to regard them as diagnostic of syphilis and give them the same diagnostic importance formerly given to the large spirochetal form alone. We are now able to demonstrate hundreds of small spirochetes in tissue-lesions in which the usual technical methods of demonstrating spirochetes show nothing at all. These small forms vary from two to eight microns in length; they are usually filamentous, with loose turns. They take the silver impregnation, as a rule, much more lightly than the large forms. They may occur singly, but often are found in crowded masses. They are either extracellular or intracellular.

Where the polymorphous form extends along the connective tissue fibers, numerous small spirochetes may be demonstrated, which re-

semble those attached to the points of the stellate shaped masses. Some of these small spirochetes are undoubtedly intracellular, but many lie between the cells or on the tissue fibers. The small spirochete form does not usually stain by the standard "Warthin-Starry" method or by other published methods used in spirochete staining; but requires the use of a specially modified technic for its demonstration. In late syphilis the small spirochete form may be present in large numbers, while there may be but few or no large spirochetes present. From the technical standpoint the small spirochete form is the most difficult to demonstrate of the various phases we have found. Small spirochetel forms, particularly the small intracellular ones, have also been demonstrated by Levaditi and his associates. They have suggested that from these and the granular forms there may be ultimately derived an ultramicroscopic form, which may be the active agent or surviving form in lesions not containing the typical spirochetes. Our technical methods, however, have the advantage over those employed by Levaditi, in that in syphilitic lesions they show the presence of these intermediate forms, so that it is unnecessary to assume the existence of a stage which cannot be demonstrated microscopically.

LYMPHOCYTIC GRANULES

In lymphocytes, and less frequently in other cells, in syphilitic lesions, there can be demonstrated by special staining methods, fine granules, which are usually arranged on one side of the nucleus, giving a crescent-shaped appearance. They are most easily demonstrated in chancres, buboes, and syphilitic lymphadenitis, and tonsillar lesions. In chancres the granules may be irregular in size, but in lymphoid tissue they are uniform. While usually associated with the large typical form of spirochete, we have found the granular form in lesions in which we could demonstrate no large forms. Very small ring forms may occur as an intermediate stage between the granule and large spirochete form, when the latter is present. The lymphocytic granules are characteristic of the early stages of syphilitic infection; after the involution of the bubo or lymphadenitis has taken place, they disappear, and the granular forms found in late syphilis are usually to be referred to the polymorphous phase, rather than to the lymphocytic granular form. Saleeby and Greenbaum^s have

recently described granules in syphilitic lymph nodes which may be the same as those we find by our special staining method. They suggest that the granules seen by them represent phagocytosis of the spirochetes by the lymphocytes; but we find the evidence of such phagocytosis to be lacking, inasmuch as the intracellular spirochetes within the lymphocytes are smaller in all dimensions than the typical forms, and may be multiple.

GIANT CELL REACTION STAGE

During the resolution of the lymph node lesion associated with the production of giant cells, granular forms and small intracellular spirochetes are present in very great numbers. The technic we have employed to show these forms is the same as that used to demonstrate the lymphocytic granular form. Each giant cell is seen to contain fine threads and granules, which occasionally take on the spiral form of the spirochete. In tonsillar syphilis, the giant cells show the same appearance as those in buboes or syphilitic lymph nodes, but in the giant cells found in the gummas of late syphilis these forms are only exceptionally present. Similar types have been described as occurring in giant cells by Levaditi and associates, who suggest again that the active agent of syphilis may be an ultramicroscopic form derived from them.

TECHNIC

The modified Warthin-Starry method, with the P_H adjustment, as advised in our previous publications,^{9, 10} still constitutes the best technical method for the demonstration of the typical large form of spirochetes, that have up to the present time been regarded as the only acceptable proof of the syphilitic nature of a given lesion. In addition to the typical spirochetal form, the method shows also some granular and atypical forms in chancre, buboes, and in other tissue lesions containing the typical spirochete form. The ring, or polymorphous form, is usually not stained, or but slightly, except in the focal aortic necroses where the ring forms and spirochetes both stain equally well. The lymphocytic granular form, and the fine granular form in the giant cell reaction stage are largely missed by the Warthin-Starry method, but are brought out by the modification given below.

MODIFICATION OF STAINING METHOD FOR THE DEMONSTRATION OF THE
INTRACELLULAR RING FORMS (POLYMORPHOUS FORMS), AND
SMALL SPIROCHETE FORMS

This method will show also the large spirochetes and all intermediate stages. The polymorphous stage extending along the connective tissue fibers is also stained, and frequently the small spirochetal forms are also brought out, although these are usually contrasted only with difficulty. Under favorable conditions, the lymphocytic granular forms may be made out. This method has the widest application in the study of the typical form of the spirochete and the variant forms.

1. Formol fixation.
2. Dehydrate. Paraffin imbedding.
3. Cut thin sections, mount on No. 1 coverslips with albumin-glycerin fixative, and dry for one to twenty-four hours at 40° to 60° C.
4. Remove paraffin in xylol, pass through alcohol to distilled water.
5. Place in $\frac{1}{4}$ per cent to $\frac{3}{4}$ per cent silver nitrate having a hydrogen-ion concentration of 4.4¹⁰ at 0° to 5° C. for one hour.
6. Develop in a mixture of yellow corn meal, which has been diluted with boiling water until it pours readily and then cooled, with 3 c.c. of 2 per cent silver nitrate to which $\frac{1}{2}$ c.c. of 5 per cent elon solution in distilled water has been added just preparatory to use. The section should turn yellow.

Other developing mixtures which may be used are:

1. 5 per cent gelatin 15 c.c., 2 per cent silver nitrate 3 c.c., and 5 per cent hydroquinone 1 c.c.
2. $\frac{1}{2}$ per cent gelatin in $\frac{1}{2}$ per cent agar 15 c.c., 2 per cent silver nitrate 2 c.c., and 5 per cent hydroquinone $\frac{1}{2}$ c.c.
3. Dilute yellow corn meal 15 c.c., 2 per cent silver nitrate 2 c.c., and 5 per cent hydroquinone 2 c.c.

METHOD FOR THE DEMONSTRATION OF THE LYMPHOCYTIC GRANULES

This modification of our staining methods shows granules, intermediate spirochetal forms, and large spirochetes in buboes, chancres, and syphilitic lymph nodes and tonsils. The fine forms in the giant cells during the period of resolution in the lymph nodes are well contrasted, but the intracellular ring form is not demonstrable, except

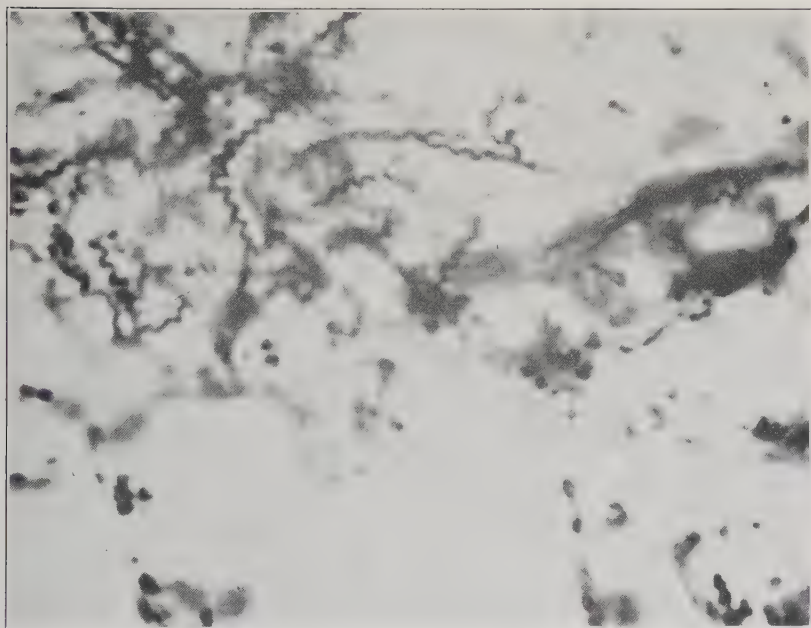


Fig. 1.—Numerous typical spirochetes with few intracellular granular forms. Early bubo. $\times 3500$.

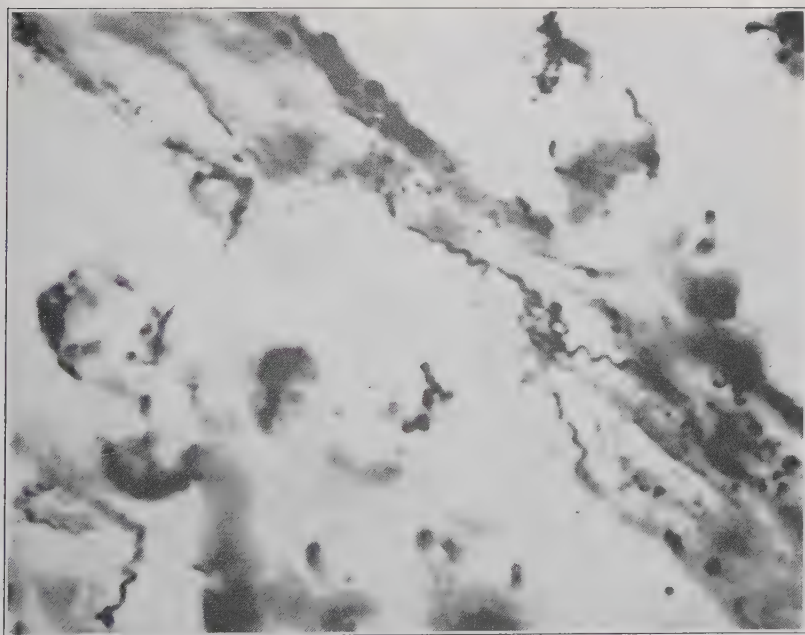


Fig. 2.—Typical spirochetes and intermediate stages in and near a capillary. Early bubo. $\times 3500$.

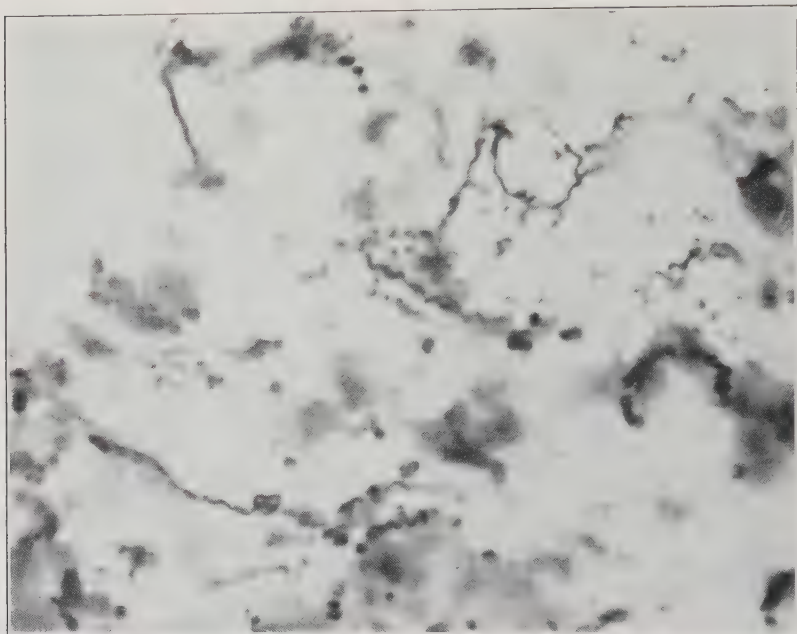


Fig. 3.—Less typical spirochetes and related granules in the lymphoid tissue. Early bubo. $\times 3500$.

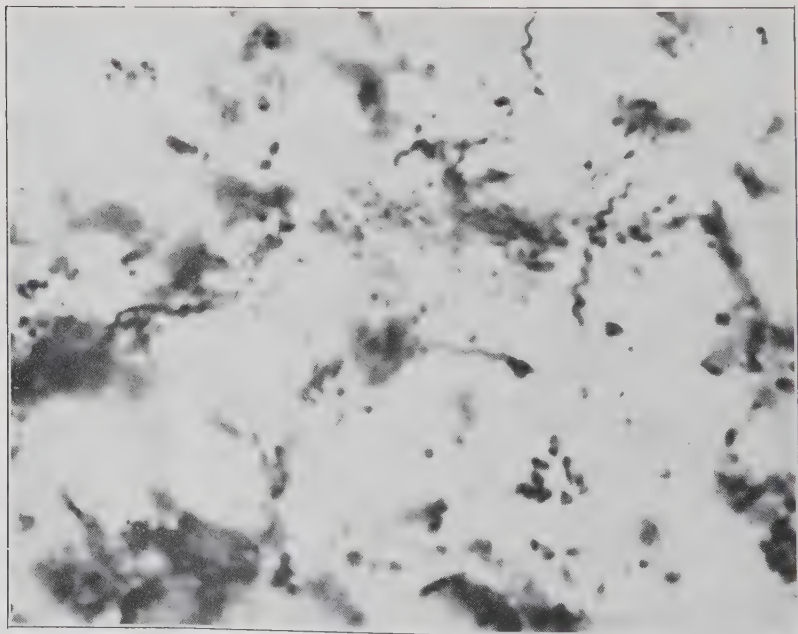


Fig. 4.—Intermediate spirochetal and granular forms. Early bubo. $\times 3500$.

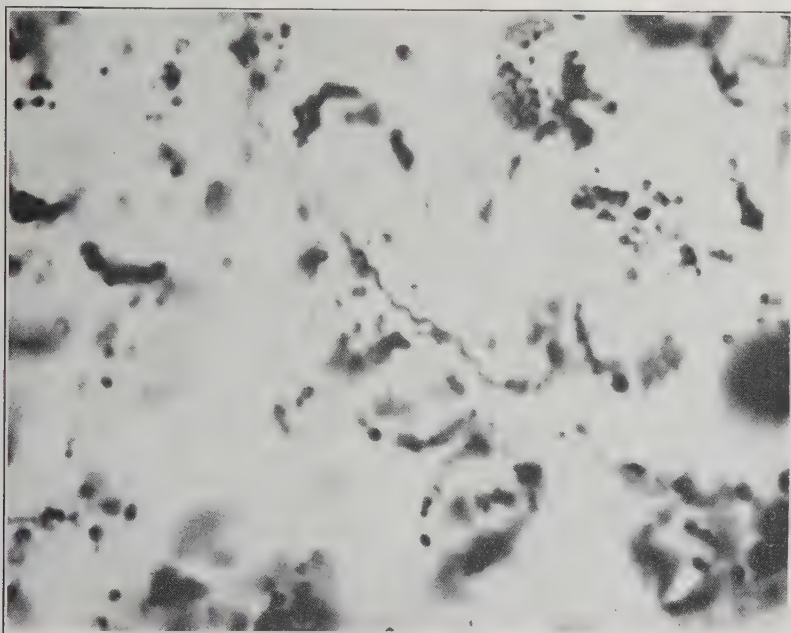


Fig. 5.—Granular and small spirochetal types with one large organism. Early bubo. $\times 3500$.

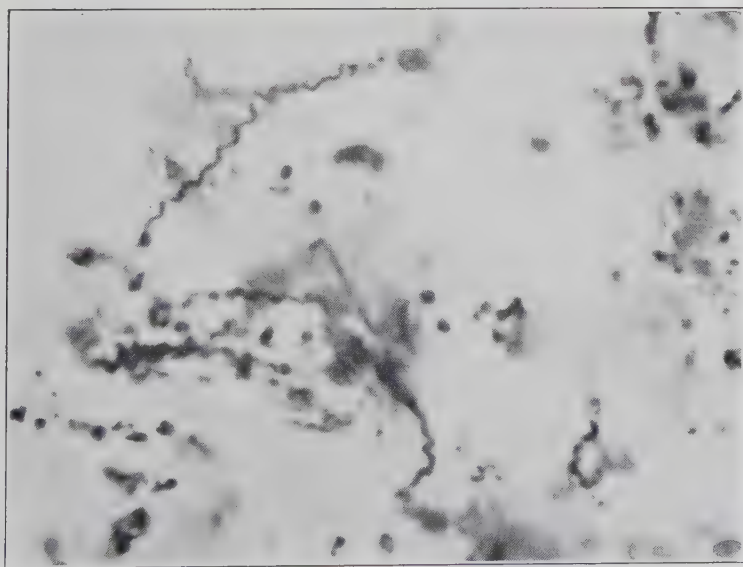


Fig. 6.—Large and small spirochetes and granules. Early bubo. $\times 3500$.

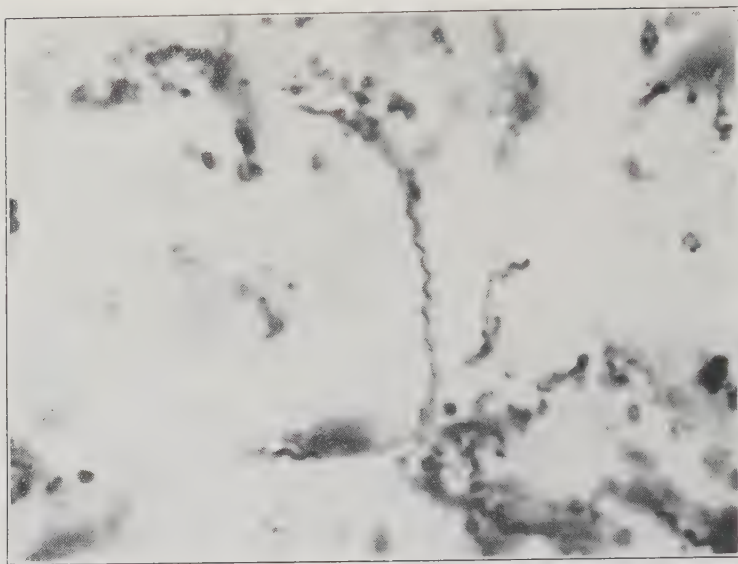


Fig. 7.—Large typical spirochete with intermediate and granular forms. Early bubo. $\times 3500$.

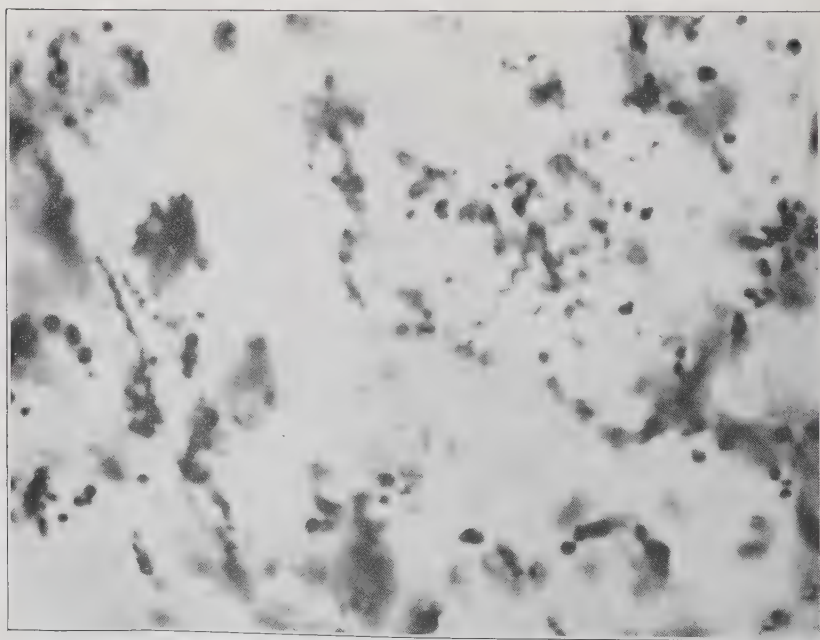


Fig. 8.—Atypical spirochetes among numerous intracellular granules. Further advanced area in early bubo. $\times 3500$.

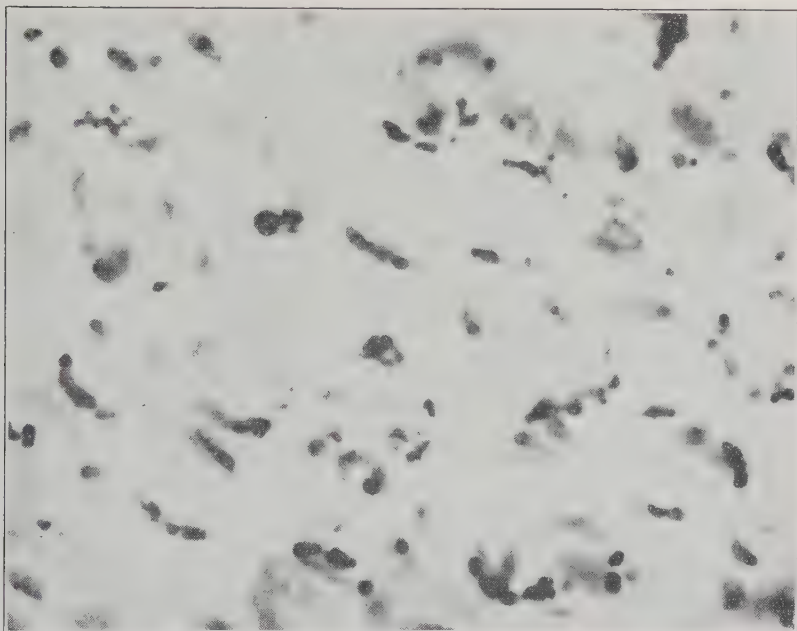


FIG. 9.—Intracellular granular forms outlining the lymphocyte walls. Advanced area in early bubo. $\times 3500$.

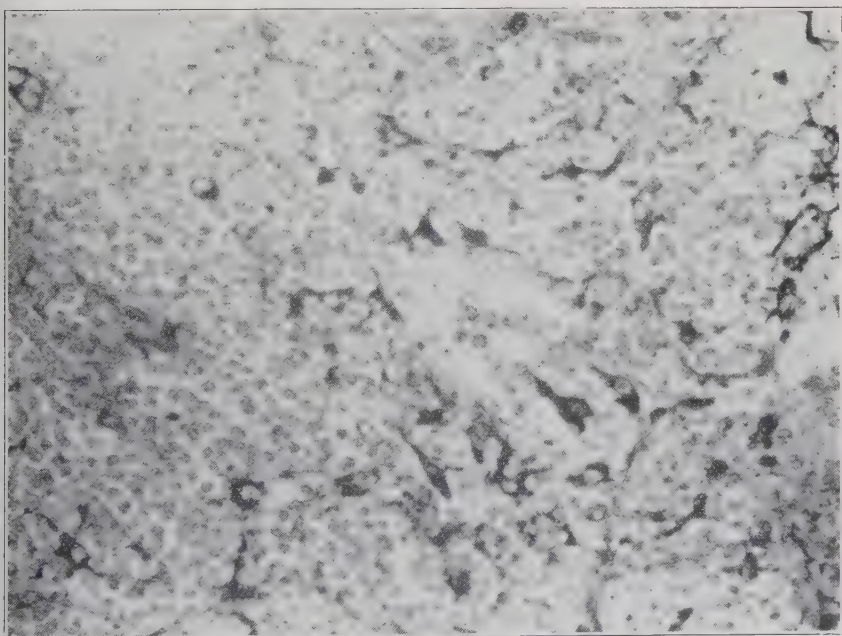


FIG. 10.—Low power view of cell types in early resolution of the bubo. Beginning of resolution in bubo. $\times 400$.

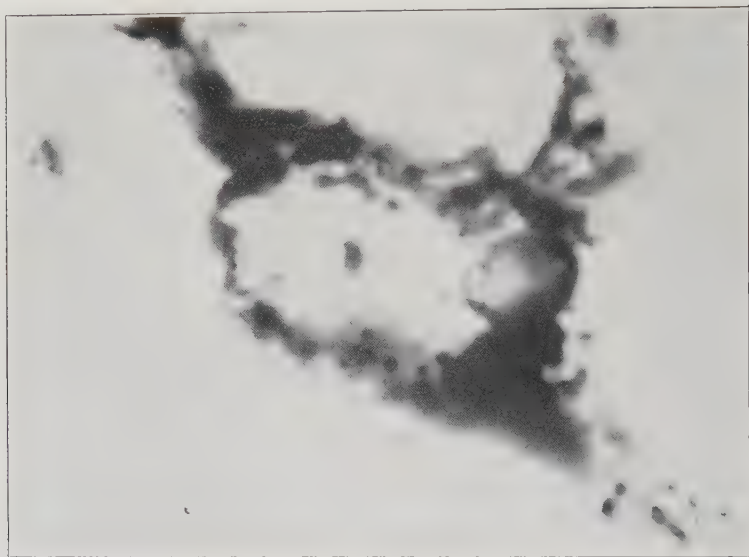


Fig. 11.—Single cell from the stage of early resolution containing numerous fine granules and filaments. Older area of bubo showing early resolution. $\times 3500$.

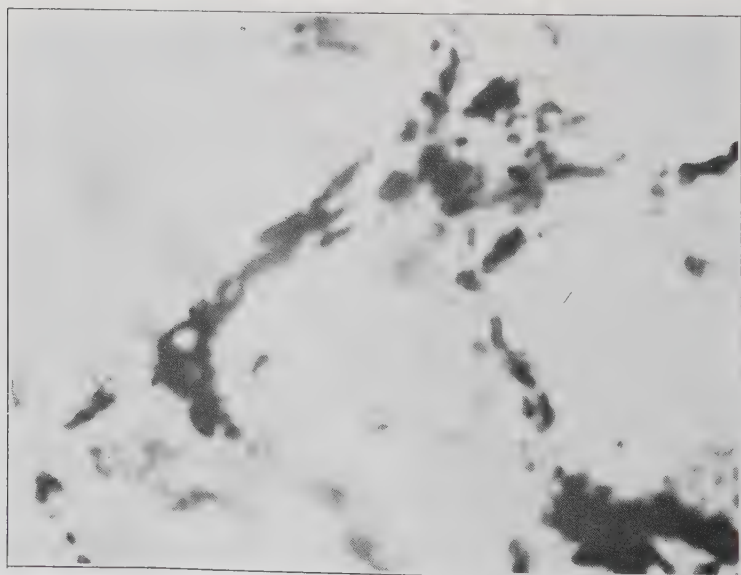


Fig. 12.—Another cell type from the early resolution period. Old area of bubo with few spirochetes. $\times 3500$.

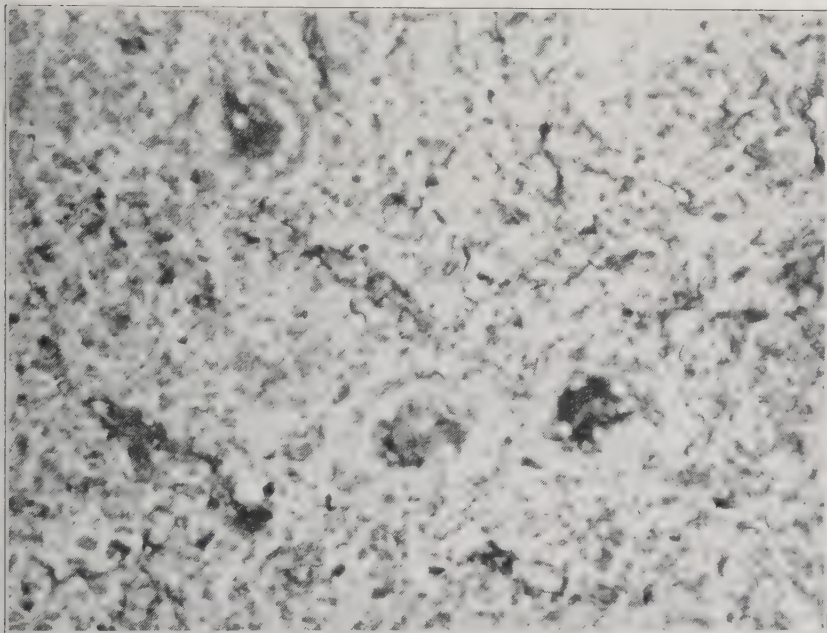


Fig. 13.—Langhans giant cell types in a later stage of resolution. Old bubo after beginning of treatment. $\times 400$.



Fig. 14.—Single giant cell showing the fine granular and irregular spiral phases. Old bubo; primary lesion healed. $\times 3500$.

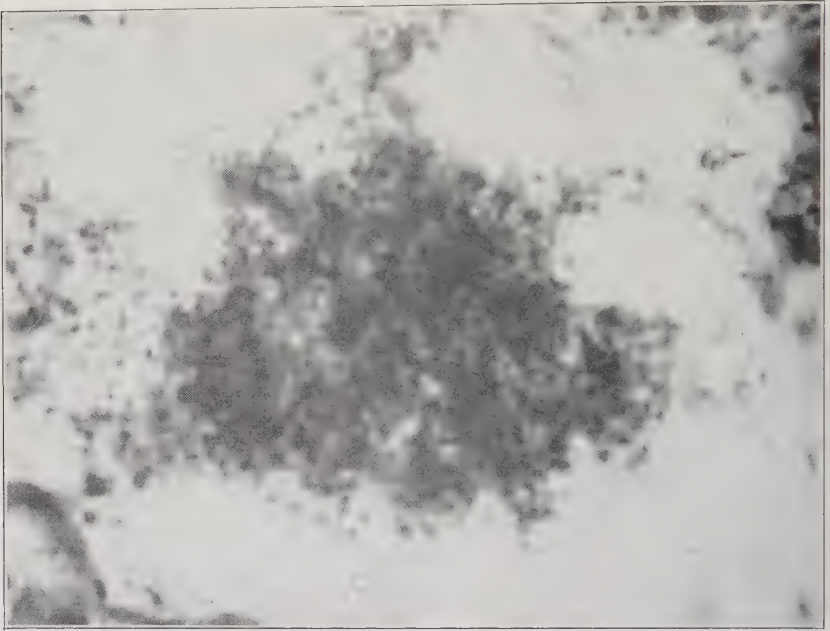


Fig. 15.—Giant cell type; the light areas at the periphery indicate the locations of the nuclei. Old bubo. $\times 3500$.

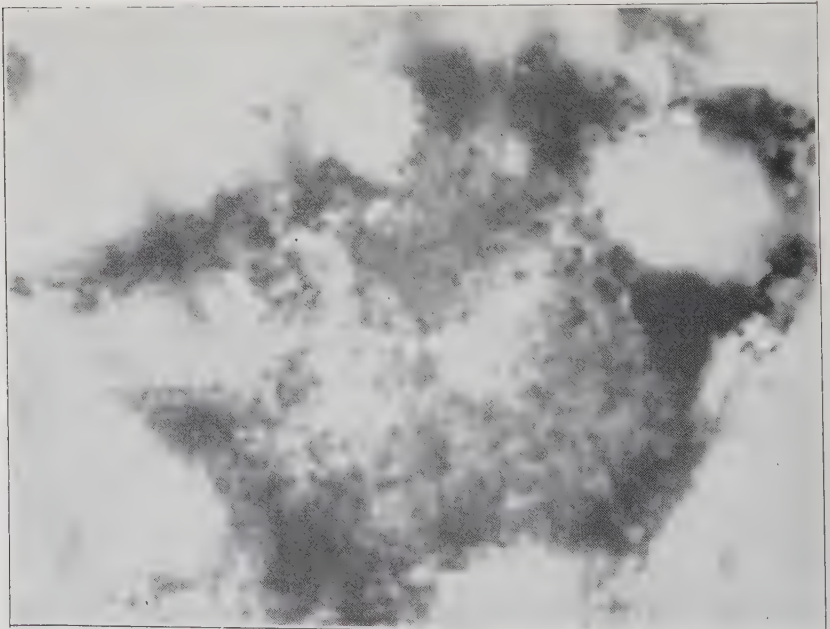


Fig. 16.—Diffuse heavily staining material in Langhans giant cell. Old bubo. $\times 3500$.

when it becomes extracellular, as in the aortic focal necroses. The value of this stain is, therefore, limited chiefly to the early stages of syphilis, and it is applicable to the study of the later stages, only under especial circumstances.

1. Formol fixation.
2. Dehydrate. Paraffin imbedding.
3. Cut thin sections, mount on No. 1 coverslips with albumin-glycerin fixative, and dry for one to twenty-four hours at 40° to 60° C.
4. Remove paraffin in xylol, pass through alcohol to distilled water.
5. Incubate at 55° to 65° C. in 2 per cent nitric acid for two hours.
6. Wash in distilled water for ten seconds or longer; incubate in 1 per cent formaldehyde gas in water, for thirty minutes, on water-bath at 80° to 90° C.
7. Wash in water (ten seconds or longer), and leave in 5 per cent formic acid or 25 per cent citric acid, for eight to ten minutes, at room temperature.
8. Pass through three changes of distilled water for two minutes, and place in $\frac{3}{4}$ per cent silver nitrate at 25° C. for one-half hour. The silver nitrate should be made up from distilled water having an adjusted P_H of 4.2, using citric acid. The silver solution should be boiled and cooled just before using. (For specific directions see Warthin-Starry method.¹⁰)
9. Develop in the following mixture, to which the ingredients are not added until time of use:

5 per cent warm gelatin 15 c.c.,
2 per cent silver nitrate 3 c.c., and
5 per cent hydroquinone 1 c.c.

Cease to develop the section by washing in distilled water when it appears light yellow.

10. Dehydrate and mount in balsam.

SUMMARY

In histologically typical lesions of syphilis there may be demonstrated by specific staining methods, certain intracellular forms, which, because of their obvious connection to typical spirochetes, may be regarded as representing phases of the syphilitic organism. Of these forms, we have described the (1) polymorphous or ring form

which may be connected to the typical spirochete or more often to the (2) fine spiral form; (3) the lymphocytic granular form with intermediate spirochetal stages; and (4) the fine threads and granules found in the giant cells. The intracellular polymorphous form and attached small spirochetes are widespread in their distribution, and apparently are found in all syphilitic lesions, being least common in areas undergoing resolution. They may occur independently of the typical large spirochete form. Their staining reaction is such that they are not usually demonstrable by the standard spirochete staining methods. The lymphocyte cell granular type is commonly found in lymphoid tissue during the early stage of syphilis, and is usually absent in the later stages. The giant-cell reaction forms are limited to the areas of resolution, and occur only occasionally in the giant cells found in the late stages or in gummas.

From the morphologic evidence it would appear that we are dealing here with intracellular stages of the *Sp. pallida*. Their relations to the typical form appear so obvious and certain; and the assumption of such clears up so many of the problems attending the demonstration of the typical spirochete in syphilitic lesions that the temptation is great to draw such conclusions. But we must take a conservative position in regard to this question, for however obvious the morphologic evidence may appear, still it can never be absolutely conclusive as to the positive nature of the relationship of apparently closely related forms. The ultimate settlement of this question must rest upon experimental evidence. In another paper we shall consider the relation of these apparently intermediary stages to histologic lesions in which it has not been possible to demonstrate the typical form of the spirochete.

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A METHOD FOR STAINING SPIRO-
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A TECHNIC, depending upon the use of phosphomolybdic acid as a mordant followed by a dye, has been developed in connection with an investigation of the morphology of *Spirocheta pallida*. While this procedure is especially applicable to morphologic research, it is so simple and dependable that we offer it as a practical method for the routine investigation of smears made from lesions suspected to be of a syphilitic nature, and also for the demonstration of moulds.

In suggesting a further staining process for spirochetes in smear preparations, it is well to consider briefly the numerous methods which have already been devised. These may be classified in three main groups: (1) the dyes, to be used with or without mordanting; (2) the metallic precipitation methods; and (3) preparations in which the background is changed in such a manner as to make the organisms visible.

Among the dye procedures are the numerous modifications of the polychrome methylene blue technic. The Romanowsky,¹ Giemsa,¹ Löffler,¹ and Wright² stains are outstanding members of the methylene blue series. Some of the other dyes which have been applied are gentian violet, basic fuchsin, thionin, dahlia, cresyl-echt-violet, Nile blue sulphate, and methyl violet.¹ Many of these have been employed in solution with carbolic acid. The fixatives and mordants which have been employed are methyl and ethyl alcohols, formaldehyde in various strengths, acetic acid, tannic acid, osmic acid, carbolic acid, chromic acid, phosphotungstic acid, potassium carbonate, sodium carbonate, and others.¹ Tilden's stain³ consists of mordanting with buffered phosphate and formaldehyde solution, and staining with gentian violet or fuchsin. These dye methods frequently give very

*An investigation conducted under a grant from the Committee on Syphilis Research.

excellent pictures but tend to be inconstant in their results, and, particularly in the case of *Spirocheta pallida*, stain the organism as a rule but palely.

The metallic methods consist of a chief group of the silver precipitations, and, as a second subdivision, all others. The outstanding silver technics are the original and improved methods of Fontana,³ Tribondeau's modification of Fontana's method,³ Stern's technic,¹ Mulzer's technic,¹ Comandon's modification of Levaditi's method,¹ and the Warthin-Starry silver agar technic.³ In addition to the use of silver precipitates, lead has also been employed in the osmic acid lead subacetate sodium sulphide procedure of Ghoreyeb.² The silver precipitation methods are more dependable than the dyes for the identification of spirochetes in smears, but from the standpoint of the study of the morphology of an individual organism are usually less satisfactory as there is a heavy coat of silver on the outside of the organism.

The third group of the methods for the demonstration of spirochetes in smears is that which Noguchi termed the "negative image method."³ This includes such procedures as the India ink method of Burri,³ the Congo red of Benians,³ and the collargol of Harrison.³ These technics are by far the least satisfactory of the three groups, and offer little either from the standpoint of practical identification or from that of morphologic studies.

THE NEW METHOD

The method suggested in the present paper is a mordant and dye combination. This was developed originally from the Alzheimer modification of Mann's eosin methyl blue stain,² which is applied to fixed tissue sections rather than to smears. The mordant is a saturated aqueous solution of phosphomolybdic acid. Any one of a number of dyes may be employed. We have found carbol iodine green, carbol-fuchsin, and Unna's alkaline methylene blue to be satisfactory. The fuchsin tends to overstain, giving results which are excellent for photographic purposes, but inferior to the iodine green for microscopic investigation where the finer detail is to be brought out. Color filters may be profitably employed in conjunction with the dyes proposed, not only for photographic purposes, but also for morphologic research under the microscope.

In smear preparations, bacteria, spirochetes, yeasts, and moulds are demonstrated. The organisms appear somewhat larger than by the

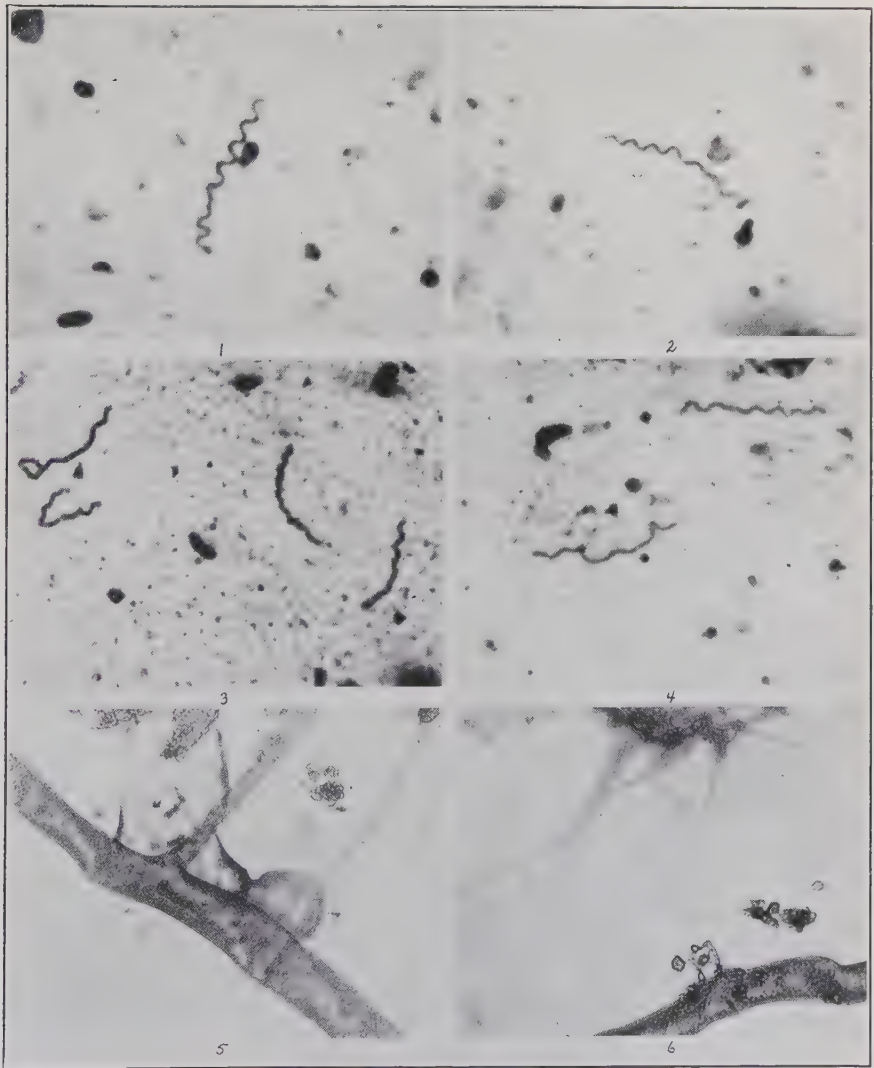


Fig. 1-6.

- Fig. 1.—Smear preparation of *Spirocheta pallida* from a rabbit chancre. Phosphomolybdic acid and carbol fuchsin. ($\times 2,650$.)
 Fig. 2.—Smear preparation showing an example of *Spirocheta pallida* from a rabbit chancre. Phosphomolybdic acid and carbol fuchsin. ($\times 2,650$.)
 Fig. 3.—Types of spirochetes from the human gum margins. Phosphomolybdic acid and carbol fuchsin. ($\times 1,700$.)
 Fig. 4.—Two spiral organisms from the human mouth. Phosphomolybdic acid and carbol fuchsin. ($\times 1,700$.)
 Fig. 5.—Extraneous mould growing on a cover slip mount of a fixed human tissue section. Phosphomolybdic acid and carbol iodine green. ($\times 170$.)
 Fig. 6.—Extraneous mould growing on a cover slip preparation. Phosphomolybdic acid and carbol iodine green. ($\times 170$.)

usual staining methods as the dye is deposited in part on the outside surface. However, unlike the silver-plating methods, it is possible to examine the interior as well as the exterior of the body. Flagella of all organisms possessing them are clearly outlined. Some of the moulds which are most easily demonstrated by this method we have not observed in any other preparations. It should be emphasized, in relation to the moulds, that when this technic is used the preparations should be recent, as old smears will be so covered with extraneous moulds as to seriously interfere with examination.

While this technic is recommended only in its application to smear preparations, it can be applied under favorable conditions to fixed tissue sections with fairly good results. A favus type of mould has been demonstrated in a skin section. On several occasions *Spirocheta pallida* has been demonstrated in tissue sections, but such results were exceptional. Also in sections from rabbit chancres it has been possible to demonstrate intracellular granular and ring forms, such as were discussed in a previous paper by Warthin and Olsen.¹ Certainly this is one of the most promising of the nonsilver technics for the investigation of the various phases of *Spirocheta pallida* (Figs. 1 to 6).

A. Phosphomolybdic Acid-Dye Method for Smears.—

1. Dry unfixed smear preparation by flaming gently.
2. Immerse in a saturated aqueous solution of phosphomolybdic acid at 50° to 65° C. for thirty to sixty minutes.
3. Wash off in distilled water for a few seconds.
4. Immerse in the staining solution at 50° to 65° C. for thirty to sixty minutes. Carbolfuchsin, carbol iodine green, and Unna's alkaline methylene blue are recommended but many other basic aniline dyes may be used.
5. Wash off in distilled water, dehydrate in absolute alcohol, transfer to xylol, and mount in Canada balsam, or after washing simply dry by blotting and mount in balsam.

B. Phosphomolybdic Acid-Dye Method for Cover Slip Sections of Fixed Tissues.—

1. Cut paraffin sections about ten microns thick and mount on clean cover glasses with albumin fixative.
2. Dry in oven at about 40° C. for two to twenty-four hours.
3. Remove paraffin in xylol, and transfer through absolute alcohol to distilled water.
4. Proceed with the method given for smear preparations.

SUMMARY

By the use of phosphomolybdic acid as a mordant, followed by various dye preparations, it is possible to demonstrate consistently bacteria, spirochetes, and moulds in smear preparations. While this method is especially recommended for morphologic investigations, it will prove of value in the routine examination of suspicious lesions and may thus supplement or replace dark-field and India ink methods. The results obtained in fixed tissue sections are irregular and undependable, but experimentally are of value.

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